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CROSSING AND CHROMOSOMAL EVIDENCE FOR TWO ADDITIONAL SIBLING SPECIES WITHIN THE TAXON *ANOPHELES DIRUS* PEYTON AND HARRISON (DIPTERA: CULICIDAE) IN THAILAND

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Abstract.—Crossing and chromosomal evidence is presented for two additional sibling species, *dirus* C and *dirus* D, within the taxon *Anopheles dirus* Peyton and Harrison, in Thailand. The affinities of the four currently recognized species in this complex in Thailand and the limitations of certain techniques used to identify the species are discussed.

Cytogenetics is one of the most useful tools for elucidating cryptic species of insects (Dobzhansky, 1970; White, 1973). The use of this method, together with biochemical, behavioral, ecological and morphological techniques has led to the recognition of a significant number of sibling species complexes of anopheline mosquitoes in different parts of the world (Bryan and Coluzzi, 1971; Kitzmiller et al., 1973; White et al., 1975; Kitzmiller, 1976; Coluzzi et al., 1979; Stegnii and Kabanova, 1978; Peyton and Harrison, 1979, 1980; Green and Miles, 1980; Subbarao et al., 1983; Green and Baimai, 1984; Green et al., 1985). The discovery of these cryptic species is a highly significant step in the development of rational and efficient control programs against the vectors of various mosquito-borne diseases.

One of the most renowned vectors of human malaria parasites in Southeast Asia is *Anopheles balabacensis* Baisas, a member of the widely distributed Leucosphyrus Group. Recently, it was demonstrated that *An. balabacensis* is a species complex (Peyton and Harrison, 1979, 1980; Baimai et al., 1981; Hii, 1982, 1984, 1985). *Anopheles dirus* Peyton and Harrison, was described as a species distinct from *An. balabacensis* in 1979, and is considered widespread in peninsular Malaysia and Thailand, while *An. balabacensis* sensu stricto is confined to the type-locality on Balabac Island and to neighboring areas of Palawan Island, Sabah and northeast Kalimantan (Peyton, unpublished data; Peyton and Harrison, 1979; Hii, 1982).

Baimai et al. (1981) recently demonstrated that the laboratory colony strain of *An. balabacensis* Perlis form from The Institute of Medical Research (Kuala Lumpur) showed different sex chromosome characters as seen in mitotic karyotype as well as on salivary gland polytene chromosomes. Genetic incompatibility between *dirus* and the *balabacensis* Perlis form also was observed (Baimai and Harrison, 1980). The recognition of the *balabacensis* Perlis form as a distinct genetic species from *dirus* was confirmed later by the detailed studies of

Table 1. Laboratory family stocks of *Anopheles dirus* complex from different localities in Thailand (otherwise indicated) used in this study.

dirus Species	Locality (Date)	No. of Isofemales Examined	No. of Families Maintained (Code)
A	Chonburi (1964) and Nakhon Ratchasima (1971)	—	Bangkok colony (BK)*
	Petchaburi (1983)	17	2 (TL)**
B	Perlis State, Malaysia (1965)	—	IMR colony (PR)*
	Trengganu State, Malaysia (1982)	5	1 (MH)
	Phangnga (1982)	2	—
C	Kanchanaburi (1980, 1982)	9	1 (KN)
D	Ranong (1983, 1984)	59	2 (RN)
	Phangnga (1982)	5	1 (PG)

* The mixed colony.
** The isofemale line now used as the reference stock for *dirus* A.

Hii (1982) who designated the Bangkok colony strain as *dirus* A and the Perlis form as *dirus* B.

In 1979–80, E. L. Peyton, at the Smithsonian Institution (personal communication) advised the authors that two additional members of the Dirus Complex existed in Thailand which could be separated from *dirus* A and *dirus* B by morphological characters. Subsequently, these members were collected and colonies of both were established in 1980–81 at the Armed Forces Research Institute of Medical Sciences (AFRIMS), Bangkok.

Recently, Wibowo et al. (1984) discovered marked differences in the amount and distribution of constitutive heterochromatin in the sex chromosomes, as revealed by the Hoechst 33258 staining technique, of specimens from a Kanchanaburi colony derived from a single isofemale of one of the two new members. Based on comparative cytological data, these investigators confirmed Peyton's morphological findings that the Kanchanaburi isoline represents a distinct genetic species designated as *dirus* C, which occurred sympatrically with *dirus* A. Additional cytogenetic studies also have revealed that the second undescribed member noted by Peyton is distinct and represents a 4th member, *dirus* D, of the complex in Thailand.

This paper presents crossing and chromosomal evidence supporting the existence of two additional sibling species, *dirus* C and *dirus* D, within the Dirus Complex in Thailand.

Materials and Methods

Individual wild caught *Anopheles* females were identified to species by morphology, isolated, and allowed to oviposit. Subsequent F_1 larvae were reared in the laboratory and used for cytogenetic confirmation of the species. Mitotic and salivary gland polytene chromosomes were prepared from 4th instar larvae using the method of Baimai et al. (1981). Isofemale lines of each cytotype were set up with respect to the X and Y chromosome configurations and maintained in the laboratory for further crossing experiments (Table 1).

The mosquitoes used in this study came from isofemale lines that were determined cytogenetically and maintained at the Department of Medical Entomology, AFRIMS, Bangkok, and at the Department of Biology, Mahidol University, Bangkok. *Anopheles dirus* A (Bangkok colony strain =

Table 2. Crossing combinations among the isolines of "*Anopheles dirus*" from different geographic origins.

Group	"dirus" Crosses ♀	"dirus" Crosses ♂	No. of Females Ovipositing (Total)	No. of Ovipositions Hatching	Mean No. of Eggs per Oviposition	% Eggs Hatching	F_1 Adults ♀	F_1 Adults ♂
1	B(MH)	B(PR)	5 (10)	5	142.2	54.0 (384/711)	168	113
	B(PR)	B(MH)	8 (10)	8	85.9	57.5 (395/687)	132	112
2	C	A (I)	6 (10)	4	59.5	47.3 (169/357)	69	73
	A	C (II)	5 (10)	3	96.0	50.0 (240/480)	112	83*
3	B(MH)	A	9 (20)	7	103.2	21.9 (203/929)	8	1*
	A	B(MH)	9 (20)	8	108.3	4.1 (40/975)	2	3*
4	B(PR)	C	8 (20)	3	54.1	10.2 (44/433)	0	0
	C	B(PR)	7 (20)	0	88.3	0 (0/618)	–	–
5	D(PG)	B(MH)	5 (10)	1	58.0	0.3 (1/290)	0	0
	B(MH)	D(PG)	0 (10)	0	0	0 (0/0)	–	–
6	D(RN)	C	2 (10)	1	209.0	12.7 (53/418)	0	0
	C	D(RN)	2 (10)	0	85.5	0 (0/171)	0	0
7	A	D(RN)	7 (10)	0	75.0	0 (0/525)	0	0
	D(RN)	A	2 (10)	2	30.0	15.0 (9/60)	8	0

* Sterile male F_1 hybrids.

BK) was used as the standard stock, whereas the IMR colony strain (Perlis form = PR) and the Trengganu colony strain (= MH) represented *An. dirus* B in this study. The isoline strain from Kanchanaburi (= KN) represents *dirus* C. Two other isoline strains of "*dirus*" were collected from Ranong (= RN) and Phangnga (= PG) in southern Thailand, and represent *dirus* D (Table 1).

Combinations of reciprocal pair-matings (Table 2) among the different cytotype strains were performed by the artificial mating technique of Ow Yang et al. (1963). In each cross pair-mating 10–20 individual females were mated with mature males. After successful copulation, each female was isolated in an oviposition vial. The number of females ovipositing, number of eggs oviposited and hatching and number of emerged F_1 adults were scored daily in the same manner as described by Klein et al. (1985). Fertility of F_1 hybrids was determined later by self-crossing among themselves as well as backcrossing to the respective parental strains (Table 3). Genetic incompatibility could be inferred on the degree of synapsis in salivary gland polytene chromosomes of F_1 larval hybrids of anoph-

Table 3. Backcrossing and selfcrossing experiments of F_1 hybrids from the crosses between isolines of *Anopheles dirus* A and *An. dirus* C.

Crosses ♀	Crosses ♂	No. of Females Ovipositing (Total)	No. of Ovipositions Hatching	Mean No. of Eggs per Oviposition	% Eggs Hatching
F_1 (I)*	A	3 (5)	3	102.0	88.6 (271/306)
F_1 (I)	C	4 (5)	4	121.3	69.3 (336/485)
A	F_1 (I)	2 (5)	2	78.0	57.1 (89/156)
C	F_1 (I)	2 (5)	2	134.5	46.8 (126/269)
F_1 (I)	F_1 (I)	5 (5)	5	189.0	76.4 (722/945)
F_1 (II)**	A	8 (12)	7	130.5	49.9 (521/1044)
F_1 (II)	C	8 (12)	7	149.5	60.1 (719/1196)
A	F_1 (II)	5 (12)	0	122.2	0 (0/611)
C	F_1 (II)	7 (10)	0	122.7	0 (0/859)
F_1 (II)	F_1 (II)	8 (12)	0	103.8	0 (0/830)

* I = cross between female *dirus* C × male *dirus* A.
** II = cross between female *dirus* A × male *dirus* C.

elines, and the degree of viability was determined by egg hatch rates.

Results

Hybridization tests.—The results of cross-mating experiments among the different sibling members of the complex are summarized in Table 2. Crosses between the 2 *dirus* B isoline strains (MH and PR), which showed the same mitotic karyotype, yielded a large number of eggs per female (85.9 and 142.2) and a high percentage of eggs hatching (54.0% and 57.5%). Adult F_1 hybrids of both sexes from both directions were fully fertile and continued to produce progeny for several generations. There was no evidence of genetic incompatibility between these two strains. The crossing evidence clearly indicates that the MH and PR strains belong to the same genetic species, *An. dirus* B.

Crosses between the *dirus* A and *dirus* C isoline strains also were successful in both directions, producing a large number of eggs (averaging 59.5 and 96.0 per female, respectively) and a high percentage of eggs hatching (47.3% and 50.0%, respectively). Many F_1 adults of both sexes were obtained in each direction. Crosses between female *dirus* C × male *dirus* A (designated as cross I) yielded fully fertile male and female F_1 hybrids. In contrast, the reciprocal cross between female *dirus* A and male *dirus* C (designated as cross II) produced fertile F_1 females, but completely sterile F_1 males (Table 2). These results indicate that genetic incompatibility exists between these two isolines, at least in one direction of hybridization. These crossing data are supported by cytological evidence described below from the F_1 hybrids.

All combinations of crosses involving the *dirus* B isoline strains (PR and MH) with the other *dirus* isolines were less successful than those matings mentioned earlier.

Crosses between *dirus* B (MH) and *dirus* A in both directions produced a large number of eggs (average of 103.2 and 108.3 per female). However, very low percentages of the eggs hatched in these crosses. Furthermore, very few adult F_1 offspring emerged, and of these, the F_1 males were sterile. Crosses between female *dirus* B (PR) × male *dirus* C were less successful because only 10.2% of the eggs hatched, and no adults emerged. The reciprocal cross between female *dirus* C × male *dirus* B (PR) gave more eggs (average of 88.3 per female), but none hatched. Furthermore, a remarkable example of genetic incompatibility was obtained from the crosses between *dirus* D (PG) and *dirus* B (MH). A very small percentage of the eggs hatched (0.3%), and only in one direction. In the reciprocal mating of this cross, none of the 10 artificially inseminated females produced eggs.

The *dirus* D isoline strains (RN and PG) exhibit similar karyotype and polytene band sequences. Unfortunately, the PG strain was lost before the RN strain was obtained, thus cross mating tests between them were not made. However, cytological evidence indicates that they are conspecific strains. All combinations of cross matings among the *dirus* D isoline strains (RN or PG) with other isolines yielded either very small numbers of F_1 female hybrids or no F_1 hybrids at all (see groups 5, 6 and 7 in Table 1). These results clearly indicate that the RN and PG isoline strains of *dirus* D were genetically distinct from the other *dirus* isolines employed in this study.

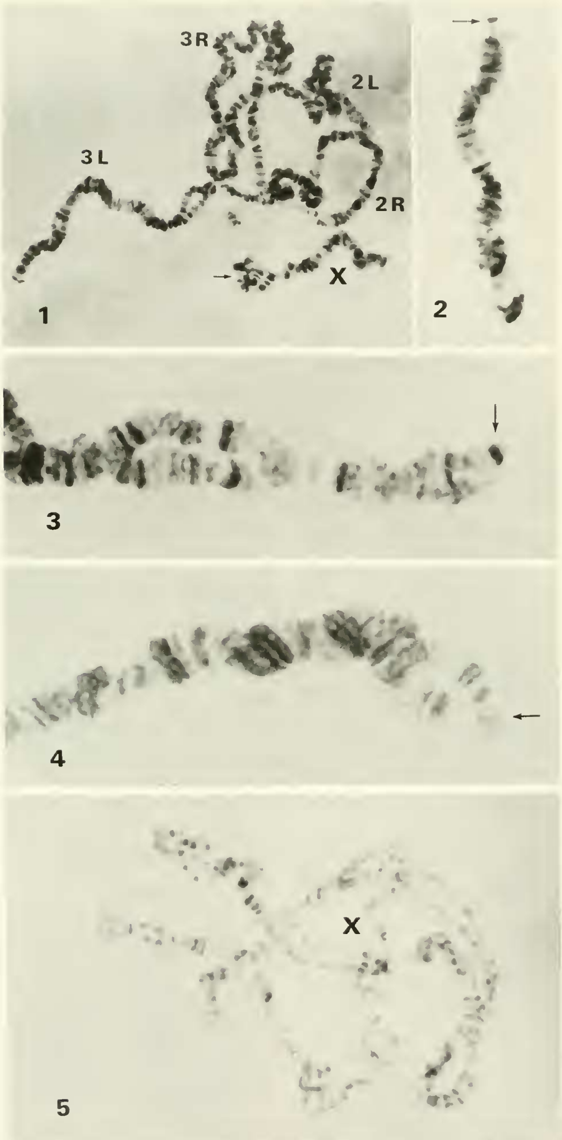
Cytological evidence.—The examination of F_1 hybrid larval salivary gland polytene chromosomes revealed some differences in banding sequences. Based on the standard salivary gland polytene chromosomes of *dirus* A (Baimai et al., 1980), the F_1 female larvae from the cross between female *dirus* A × male *dirus* C exhibited approximately 5–10% asynapsis of the chromosome complement (Fig. 1). In addition, marked differences in polytene banding sequences were observed at zone 6 (Fig. 1, arrow) and at the tip of the X chromosome (Fig. 2, arrow), as well as at the tips of chromosome arm 2L and arm 2R (Figs. 3, 4, respectively). These differences are good chromosome markers for the *dirus* C karyotype.

Larval salivary gland polytene chromosomes of F_1 hybrid females from the cross between female *dirus* A × male *dirus* B (PR) showed approximately 80% asynapsis of the chromosome elements (Fig. 5). This suggests that genetic differentiation at a submicroscopic level between these two species is more extensive than in the case of *dirus* A and *dirus* C.

The chromosome complement of the *dirus* D (RN and PG) strains is remarkably different from the standard *dirus* A colony strain. The F_1 female larval chromosomes from the cross between female *dirus* A × male *dirus* D (RN) showed over 90% asynapsis along the 5 chromosome arms (Fig. 6). The X chromosome of the *dirus* D (RN) strain exhibited a fixed inversion covering zones 1 and 3 of the X chromosome compared with the standard sequence of the *dirus* A strain (Fig. 7). Zone 6 of the X chromosome of the F_1 female hybrids was asynapsed completely. Moreover, asynapsis in chromosome arm 2R of F_1 hybrids in this case was more pronounced (Fig. 8) than in the case of *dirus* C × *dirus* A F_1 hybrids. Overall, asynapsis was a persistent feature of the hybrid polytene chromosomes of the Dirus Complex. In addition, an analysis of the mitotic karyotype showed that the X and Y chromosomes of the *dirus* D (RN) strain are shorter than those of *dirus* A as can be observed in F_1 hybrid larval chromosomes (Figs. 9, 10). Thus, the cytological observations clearly support the sterility and viability evidence from the hybridization experiments described above.

Discussion

Recent morphological, genetic and cytogenetic studies of the taxon *dirus* have revealed that it consists of at least 3 genetic



Figs. 1–5. Figs. 1–4. Larval salivary gland polytene chromosomes of F_1 hybrid females from the cross matings between female *dirus* A × male *dirus* C. 1, Condition of synapsis along the 5 chromosome elements, zone 6 of the X chromosome (small arrow) is almost totally asynapsed. 2, Complete synapsis of zones 1–5 of the X chromosome with a distinct banding difference at the tip (arrow). 3, 4, Tips of chromosome arm 2L and arm

species namely *dirus* A, B and C (Peyton, unpublished data; Baimai et al., 1981; Hii, 1982; Wibowo et al., 1984). The present investigation confirms those findings. Further, the present cytogenetic evidence has confirmed the fourth species recognized morphologically by Peyton (unpublished) within this taxon. Provisionally this species is designated *dirus* D, and it is represented by the RN and PG isoline strains from southern Thailand populations. Our results, however, seem to be in disagreement with the interpretation of Kanda et al. (1981). Based on their hybridization data, Kanda and co-workers are of the opinion that their colony strains from Chantaburi, Kanchanaburi and IMR only represent geographical populations of *An. balabacensis.*

The results of our cross mating experiments clearly indicate that *An. dirus* is actually a cluster of closely related species. The divergence of these siblings could have occurred comparatively recently. The present data suggest that *dirus* A and *dirus* C are very closely related and they occur sympatrically, at least in Kanchanaburi province. Artificial mating between them is possible under laboratory conditions. Whether gene flow between these two genetic species occurs in nature is not known. The species isolating mechanism for these two siblings probably involves premating isolation, as well as the unidirectional genetic incompatibility detected in this study.

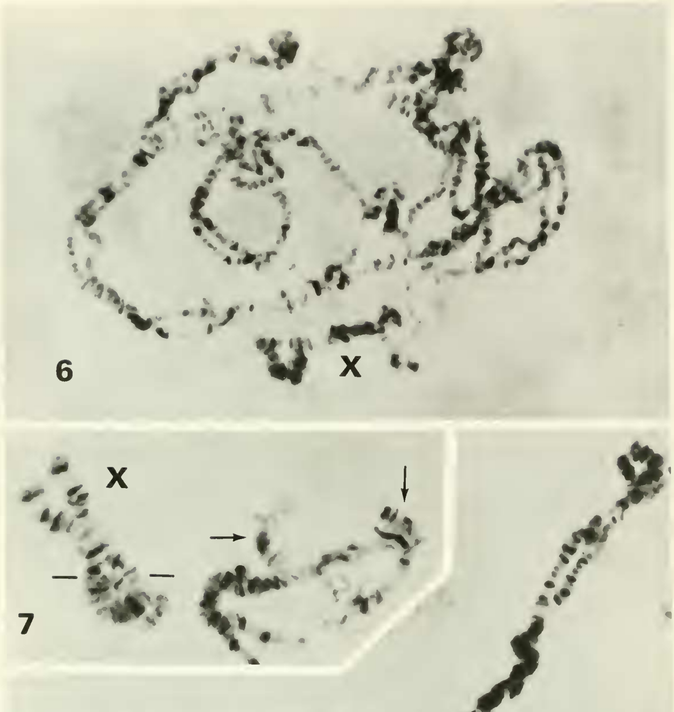
Both *dirus* A and *dirus* C exhibit similar banding sequences of salivary gland polytene chromosomes and general mitotic karyotypes, although the former shows heterochromatin variation in the sex chromosomes (Baimai et al., 1984). However, striking differences in the polytene chromosomes of F_1 hybrids were observed at the tips of chromosome X and chromosome arms 2L and 2R. Furthermore, differences in the amount of heterochromatin are noticeable in the sex chromosomes (Wibowo et al., 1984). In general, the mechanism in the process of species differentiation for siblings in the Dirus Complex resembles that for some species groups of the picture-winged Hawaiian *Drosophila* (Ohta, 1980; Carson and Yoon, 1982).

Anopheles dirus B (= Perlis form) is genetically distinct from *dirus* A and *dirus* C. Hybridizations between *dirus* B and *dirus* C were cross-sterile, producing no adult F_1 hybrids in either direction. Cross mating between *dirus* B and *dirus* A, however, yielded very few adult F_1 hybrids, of which the males were completely sterile. *Anopheles dirus* B showed cytological differences from *dirus* A both in salivary gland polytene chromosome (Fig. 5) and mitotic karyotype (Baimai et al., 1981). So far, distribution records indicate that *dirus* B is confined to southern Thailand. Thus, *dirus* B may be isolated geographically from *dirus* A. On the other hand, *dirus* A seems to be widespread in central and northern Thailand. We now are investigating the boundary limits of *dirus* A and *dirus* B in southern peninsular Thailand. The distribution of *dirus* C is limited to collection areas in Kanchanaburi Province, in western Thailand where it coexists with *dirus* A and *dirus* D, and an isolated questionable area in southern Thailand where it may be sympatric with *dirus* B.

The highest degree of genetic incompatibility was found in all combinations of hybridization tests involving *dirus* D. Most cross matings involving the RN or PG strains of *dirus* D completely failed to produce F_1 hybrid adults. However, cross matings between female *dirus* D (RN) × male *dirus* A produced a few F_1 hybrid females which were very weak. Asynapsis in F_1 lar-

←

2R, respectively (chromosomes of *dirus* C are indicated by arrows). Fig. 5. Larval salivary gland chromosome elements of a F_1 hybrid female from a cross between female *dirus* A × male *dirus* B (PR) showing extensive regions of asynapsis.



Figs. 6–10. Salivary gland polytene chromosomes and mitotic karyotypes of F_1 hybrid larvae from crosses between female *dirus* A × male *dirus* D (RN). 6, Asynaptic condition of the whole polytene chromosome complement. 7, A fixed inversion difference on the distal half and the complete asynaptic region of zone 6 of the X chromosome (arrows). 8, Asynapsis in chromosome arm 2R. 9, Comparison of heterochromatin differences of the X chromosomes in a F_1 female larval neuroblast cell. 10, A short Y chromosome of *dirus* D compared with the X chromosome of *dirus* A in a F_1 hybrid male.

val polytene chromosomes was extensive, covering more than 90% of the chromosome elements. Our data indicate that *dirus* D is genetically remote from the other siblings in the Dirus Complex. *Anopheles dirus* D apparently is distributed widely in central and southern Thailand, and northern Malaysia, and has been found in sympatry with *dirus* A, *dirus* B and *dirus* C.

The use of heterochromatic variation in sex chromosomes as a means for routine identification of our material from the field has distinct limitations. On the other hand, analysis of salivary gland polytene chromosomes now provides a better means of species identification of this sibling species complex than mitotic karyotypes, and this method is used routinely in our laboratory. First, X chromosome heterochromatin variation cannot be used routinely because it is difficult to score and is seen only in rare, superb preparations. The Y chromosome variation, on the other hand, is scored much more easily and is available for routine identification of families from wild-caught material. There is a quantitative difference in data from these two sources of variation. The X chromosome data can provide direct evidence for gene flow characteristics in nature and thus, evidence for mixtures of cryptic species in samples. The Y chromosome data cannot provide such evidence because of the combination of the obvious hemizygous condition of the Y in males and the knowledge that most female anophelines are mated successfully only once. Consequently, different Y chromosomes are not expected to occur together in single broods, and we cannot tell from their distribution in broods whether these Y chromosomes represent intra- or interspecific variation. The best we can do is to correlate Y chromosome variation with primary evidence for the different species within the Dirus Complex. The most widely used criterion is interspecific sterility as seen in laboratory crossing experiments.

Our procedure has been to score Y chromosome variation in samples from nature and where a sample or sub-sample of families shows the same Y chromosome, cross one of these families to laboratory reference stocks. At first, we were forced to use the non-isoline colonies of the Bangkok strain (species A) and the Perlis form (species B). As identified isofemale lines became available, we replaced the non-isoline colonies as reference stocks and also established isofemale lines as reference stocks for species C and D.

The recognition of the existence of cryptic species within the taxon *An. dirus* has led to a better understanding of the process of species differentiation of the Leucosphyrus Group of *Anopheles*. Further information on species distributions, behavior and population dynamics of these siblings undoubtedly will lead to a better understanding of malaria transmission and strategies for effective vector control in this region. Differences in biological properties and behavior with respect to the vectorial capacity and the epidemiological significance of the four member species of the Dirus Complex are under investigation. Presently, the identification of these genetic species from natural samples is a problem. A practical taxonomic key is now being developed at the Walter Reed Biosystematics Unit (Peyton, personal communication). Another technique which may be valuable in identifying these species is recombinant DNA for species specific DNA probes.

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